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Identification of Nontuberculous Mycobacteria Existing in Tap Water by PCR-Restriction Fragment Length Polymorphism

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This paper presents the finding of the possible cause of the high false-positive rate in acid-fast staining in histological examinations. Using acid-fast staining, culture, and PCR, acid-fast bacilli were detected in 83.7% of 49 hospital tap water samples and nontuberculous mycobacteria (NTM) were detected in 20.4% of the same 49 samples. The 10 NTM isolates were also identified to the species level using PCR-restriction fragment length polymorphism. Our findings indicate that NTM in hospital tap water are the possible cause of false positives in acid-fast staining and of nosocomial infection in immunocompromised patients.

Nontuberculous mycobacteria (NTM) are the common organisms which cause diseases in immunocompromised patients. *Mycobacterium genavense* from hospital tap water has been demonstrated to cause nontuberculous mycobacteriosis in human immunodeficiency virus-infected patients (5). In addition, several kinds of NTM have been identified in ice samples and public drinking water samples (4). Molecular techniques, including PCR and restriction fragment length polymorphism (RFLP), have been applied for the detection and identification of mycobacteria, including *M. tuberculosis* and NTM. Use of the genus-specific 16S rRNA and the *rpoB* gene of mycobacteria for the identification of NTM has also been documented (2, 6). In addition, other genes, such as those encoding the conserved and nonconserved regions in 65-kDa heat shock proteins of mycobacteria, have been found to be suitable for the identification of NTM (9).

In our clinical laboratory, the contamination of tap water by NTM usually causes false positives in the acid-fast staining of histological sections. This causes a major problem in the quality control of clinical examinations, especially for histological examinations. Thus, the detection of mycobacteria in tap water was performed. The tap water samples were subjected to acid-fast staining, Lowenstein-Jensen (LJ) medium culture, and the PCR-RFLP method to detect NTM. The gene for heat shock protein 65 was chosen for the detection of NTM by PCR in this study. The positive result of PCR amplification generated a 439-bp fragment, which was further digested by restriction enzymes for the identification of NTM to the species level. Our results should be important for quality control of acid-fast staining in histological examinations and for the investigation of nosocomial infection in immunocompromised patients.

Tap water samples (200-ml volume) were collected from various sources in the clinical laboratory at Yuan's General Hospital. After centrifugation at 4,500 × *g* for 30 min, the pellet was resuspended in 60 μl of Tris-EDTA (TE) buffer. All water samples were subsequently examined by acid-fast staining, LJ medium culture, and direct-PCR analysis.

Acid-fast staining was performed by the traditional Kinyoun method (7). The glass slide was cleaned with 95% ethanol and then coated with bovine serum albumin (fraction V; Sigma Chemical Co., St. Louis, Mo.). Each water sample (10 μl) was applied. The acid-fast stain reagent was Bacto TB Stain Set K (Difco Laboratories, Detroit, Mich.).

For LJ medium culture, each 10-μl water sample was inoculated onto LJ medium (Difco Laboratories) and then cultured at 37°C. The growth of the cultures was examined every day for 4 weeks. When colonies appeared on the LJ medium, they were subjected to acid-fast staining and indirect PCR analysis if acid-fast positive.

For direct-PCR amplification, 10 μl of pellet from tap water was used for DNA extraction. For indirect-PCR amplification, one loopful of a colony was suspended in 1 ml of TE buffer. In both cases, the DNA was extracted by boiling the sample for 30 min to lyse the mycobacteria (11). After centrifugation, the supernatants were used for PCR amplification. The primers used in our experiments were those designed by Telenti et al. (9). The forward primer for PCR was Tb11, 5′-ACCAACGATGGTGTGTCCAT-3′, and the reverse primer was Tb12, 5′-CTTGTCGAACCGCATACCCT-3′. PCR was performed in 50-μl reaction mixtures consisting of 5 μl of DNA template, 0.2 mM deoxynucleoside triphosphates, 0.5 μM primers, and 1.25 U of *Taq* polymerase (Takara Biomedical Co., Ltd., Shiga, Japan). The PCR conditions were 95°C for 5 min followed by 45 cycles of 95°C for 1 min, 56°C for 1 min, and 72°C for 1 min and then extension at 72°C for 5 min (GeneAmp PCR system 9600; Perkin-Elmer Instrument, Norwalk, Conn.). The PCR products were analyzed on 2% agarose gels. The gels were then stained with ethidium bromide and photographed. The positive control for PCR in our experiments was *Mycobacterium gordonae*, which had been identified previously in our laboratory.

The restriction enzymes *Bst*EII and *Hae*III were used for the restriction fragment analysis of PCR products, as described by Telenti et al. (9). The digestions were performed for 1 h at 37°C and then the products were separated on 2% agarose gels for RFLP analysis. The restriction enzymes and the molecular

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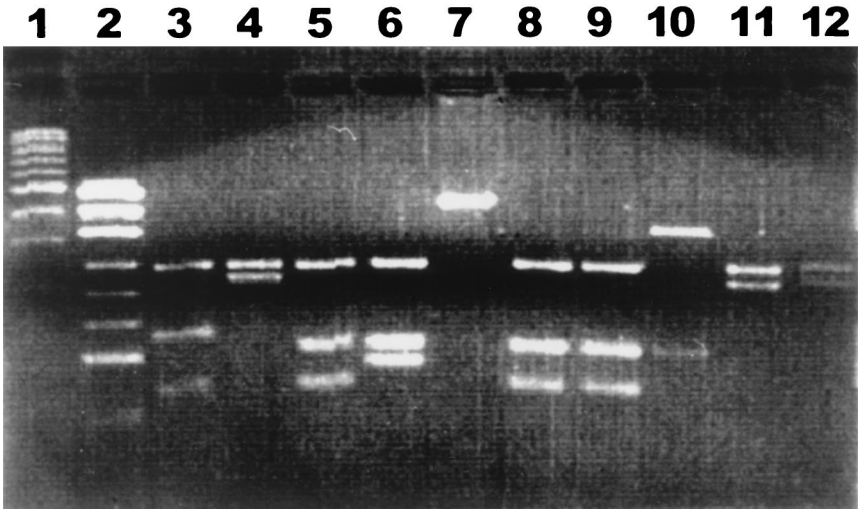

FIG. 1. Restriction analysis of PCR products with *Bst*EII enzyme. Lane 1, molecular size markers of 100-bp ladder; lane 2, pUC19 digested by *Msp*I, including 501 (489)-, 404-, 331-, 242-, 190-, 147-, 111 (110)-, and 67-bp bands; lanes 3 to 12, restriction fragments of PCR products obtained by digestion with *Bst*EII and then measured by 2% agarose gel electrophoresis.

size markers used in this study, *Msp*I-digested pUC19 fragments and 100-bp DNA ladders, were from MBI Fermentas Inc. (Lithuania, Italy).

Forty-nine samples from different sources of tap water in Yuan's General Hospital were examined in this study. The samples were concentrated from 200 ml of tap water by centrifugation, and then the pellets were resuspended in 60 μl of TE buffer. The concentrated samples were subjected to three examinations: acid-fast staining, LJ medium culture, and PCR-RFLP. Each examination used 10 μl of the concentrated sample, representing 33.3 ml of the original tap water sample, to detect the existence of NTM in the tap water. The result shows that 41 of the 49 tap water samples examined (83.7%) contained acid-fast bacilli by acid-fast staining.

None of the 49 tap water samples examined was positive for NTM by direct PCR-RFLP. After culture on LJ medium for several days, colonies appeared from 13 samples. To confirm that the 13 isolates on LJ medium were NTM, indirect PCR was performed. Ten samples were identified as NTM. The specific 439-bp PCR products were obtained from 10 samples (data not shown). Therefore, combining LJ medium culture and PCR assay, 10 out of the 49 samples examined (20.4%) were determined to contain NTM.

In accordance with the previous study by Telenti et al. (9), the 10 NTM isolates with 439-bp PCR products were then treated with *Bst*EII and *Hae*III for identification to the species level. As shown in Fig. 1 and 2, respectively, there were six different RFLP profiles produced by digestion with *Bst*EII (439, 325/125, 245/220, 245/140/85, 245/125/100, and 245/125/80) and eight different RFLP profiles produced by digestion with *Hae*III (200/135, 170/115, 140/105/70, 140/120, 140/105, 150, 150/135, and 155/135/95).

From the six kinds of *Bst*EII and eight kinds of *Hae*III RFLP patterns, 9 of 10 samples of NTM were identified to the species level. Six species of mycobacteria were identified, including one *M. gordonae* type I, one *M. gordonae* type II, one *M. gastri*/*M. kansasii*, two *M. fortuitum* subsp. *3rd variant*, one *M. simiae*, two *M. scrofulaceum*, and one *M. szuigai* (Table 1). One sample (Fig. 2, lane 10) presented a profile different from the known RFLP profiles for NTM species and could not be identified.

The tap water samples from Yuan's General Hospital were examined using acid-fast staining, LJ medium culture, and PCR-RFLP. Three results were obtained. First, 41 of 49 tap water samples examined (83.7%) contained acid-fast bacilli. Second, 10 of 49 tap water samples examined (20.4%) contained culturable NTM. Third, PCR-RFLP analysis of the nine culture-positive samples identified them as six species of NTM, i.e., two *M. gordonae*, two *M. fortuitum*, two *M. scrofulaceum*, one *M. kansasii-M. gastri*, one *M. simiae*, and one *M. szuigai*.

In our study, the detection rate of NTM from tap water by LJ medium culture was 20.4%. Covert et al. (4) have shown that NTM were detected in 54% of ice samples and 35% of public drinking water samples but not in bottled water or cisterns. Additionally, Argueta et al. (1) showed that 25 of 121 food samples were positive for NTM (20.6%). Therefore, the incidence of NTM in our tap water samples is similar to the incidences in these other environments.

Interestingly, NTM existed in 20.4% of tap water samples but acid-fast bacilli were detected in 83.7% of tap water samples. The difference between these two examinations, 63.3%, is suggested to represent acid-fast bacilli other than mycobacteria. Perhaps other acid-fast bacilli, such as *Nocardia* spp. identified by others (3), also exist in tap water. However, this possibility remains to be further examined and demonstrated.

Among 10 positive samples, six species of NTM were identified. Argueta et al. (1) also isolated six different species of NTM from food samples, but the predominant species was *M. avium*. *M. avium* was not identified in this study. Perhaps, the difference in the diversity of species between the two studies is associated with the exposure of the samples to the different environments.

Contamination by NTM in tap water or other water supplies is generally known to cause nosocomial-infection problems in public health. Also, *M. genavense* in hospital tap water was demonstrated to cause nontuberculous mycobacteriosis in hu-

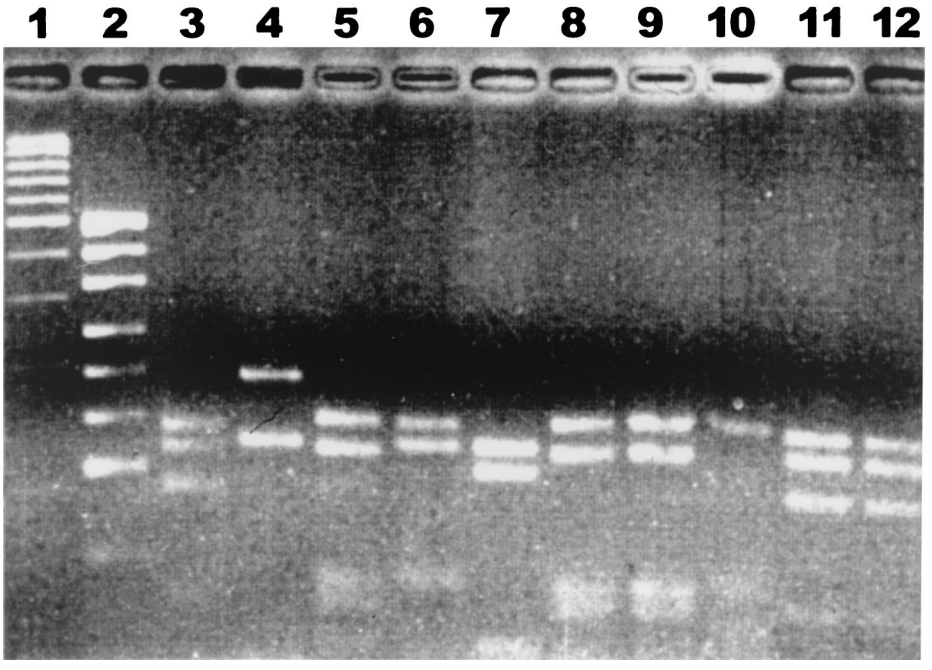

FIG. 2. Restriction analysis of PCR products with *Hae*III enzyme. Lane 1, molecular size markers of 100-bp ladders; lane 2, pUC19 digested by *Msp*I, including 501 (489)-, 404-, 331-, 242-, 190-, 147-, 111 (110)-, and 67-bp bands; lanes 3 to 12, restriction fragments of PCR products obtained by digestion with *Hae*III and then measured by 2% agarose gel electrophoresis.

man immunodeficiency virus-infected patients (5). The nosocomial outbreaks and pseudo-outbreaks caused by NTM have been indicated to be associated with the contamination of hospital water supplies (10). In addition, several species of mycobacteria, including *M. gordonae*, *M. flavescens*, *M. chelonae*, and *M. simiae*, were also identified in cooling and spray water samples in dental units (8). Therefore, the tap water and other hospital water supplier should be examined for NTM to prevent nosocomial infection. We recommend that the water used for histological examinations and for immunocompromised patients be filtered.

In conclusion, 49 tap water samples were examined by acid-fast staining, LJ medium culture, and PCR-RFLP. The results indicated that 41 of 49 tap water samples examined (83.7%) contained acid-fast bacilli and 10 of 49 tap water samples examined (20.4%) contained NTM. The nine NTM isolates were further identified to the species level. These findings indicate that NTM in hospital tap water are the possible cause of false positives in acid-fast staining and of nosocomial infection in immunocompromised patients.

TABLE 1. RFLP patterns and species identifications of culture-positive tap water samples

RFLP pattern		NTM	No. of samples (n = 10)
*Bst*EII	*Hae*III		
439 (no digestion)	140/105	*M. szuigai*	1
325/125	150	Unidentified	1
245/220	200/135	*M. simiae*	1
245/220	155/135/95	*M. scrofulaceum*	2
245/140/85	140/105/70	*M. gastri*/*M. kansakii*	1
245/125/100	140/120	*M. gordonae* Type II	1
245/125/80	150/135	*M. fortuitum* subsp. *3rd variant*	2
245/125/80	170/115	*M. gordonae* Type I	1

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